



Staurosporine induces a neuronal phenotype in SH-SY5Y human neuroblastoma cells that resembles that induced by the phorbol ester 12-*O*-tetradecanoyl phorbol-13 acetate (TPA)

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Treatment of SH-SY5Y human neuroblastoma cells with the protein kinase inhibitor staurosporine, induced both morphological and functional differentiation in these cells. The effects of staurosporine were comparable to those induced by the protein kinase C (PKC) activator, 12-*O*-tetradecanoyl phorbol 13-acetate (TPA), with respect to induction of neuronal differentiation, i.e. neurite outgrowth, inhibition of DNA synthesis, induction and down-regulation of *c-myc* protein expression, induction of mRNA for both neuropeptide Y (NPY) and growth associated protein 43 (GAP-43) and stimulation of tyrosine hydroxylase expression. Staurosporine failed to translocate PKC to the membrane fraction or to stimulate phosphorylation of the endogenous PKC substrate *M*, 80,000 (p80). Instead, staurosporine inhibited TPA-induced phosphorylation of p80.

Staurosporine; Protein kinase C; Neuroblastoma differentiation; Neuropeptide Y; Tyrosine hydroxylase; GAP-43; *Myc*

1. INTRODUCTION

SH-SY5Y human neuroblastoma cells differentiate to mature neuronal cells when treated with the phorbol ester, 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) [1,2]. The actions of tumour promoting phorbol esters have been shown to be mediated by protein kinase C (PKC) [3]. The role of PKC in differentiation of SH-SY5Y cells is unclear at present. H-7, an inhibitor of the enzyme, failed to block the TPA-induced maturation of SH-SY5Y cells but instead potentiated neurite outgrowth induced by the phorbol ester in these cells [4]. Down-regulation of PKC, on the other hand, inhibited the TPA-induced differentiation [5].

Staurosporine, a microbial alkaloid, is a potent inhibitor of protein kinases, including PKC [6,7]. In the present study we have compared the effects of staurosporine with those of TPA on morphological differentiation, induction and down-regulation of *c-myc* protein, stimulation of neuropeptide Y (NPY) and growth-associated protein (GAP-43) mRNA expression and induction of tyrosine hydroxylase protein expression in SH-SY5Y cells. Effects of staurosporine on intracellular distribution of PKC as well as on endogenous PKC substrate *M*, 80,000 (p80) phosphorylation were also studied.

2. MATERIALS AND METHODS

2.1. Antibodies

Monoclonal antibody to *c-myc* protein (9E10) [8] was a kind gift from Dr. K. Alitalo (Dept. of Virology and Pathology, Univ. of Helsinki, Finland) and panmyc polyclonal antibody was from Cambridge Research Laboratories (Cambridge, UK). The monoclonal antibody against PKC was from Amersham (UK). The polyclonal antibody against tyrosine hydroxylase was purchased from Boehringer-Mannheim (Germany).

2.2. Cell cultures

SH-SY5Y cells [9] were donated by Dr. June Biedler (Sloan Kettering Institute, New York, USA). The cells were cultured as previously described [4].

2.3. Assay of DNA synthesis

Cells were seeded at a density of $6-8 \times 10^4$ per 5 diameter Petri dish. Staurosporine (Kyowa Hakko, Tokyo, Japan) or TPA was added 24 h after plating. DNA synthesis was assayed by incubating the cells in the presence of 0.5 μ Ci/ml of [³H]thymidine for 2 h. The cells were washed and counted for radioactivity as described in [5].

2.4. Northern blotting

Northern analysis of poly(A⁺) RNA was performed essentially as previously described by random prime labelled cDNA probes [5]. The cDNA for the coding region of human GAP-43 was generated by PCR (Örnöft E., Beutzholtz, C., Pahlman S. and Hammerling U., to be published). The probes for human NPY [10] and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [11] were from Drs. Dan Larhammar and Ray Wu, respectively.

2.5. Western blotting

For analysis of *myc* proteins, SH-SY5Y cells were cultured for 24 h prior to incubation with 10 nM TPA or 25 nM staurosporine. Nuclei fractions were prepared as described in [12] by lysing the cells on ice for 5 min in 20 mM HEPES (pH 6.8), 5 mM potassium chloride, 5 mM magnesium chloride, 0.5% Nonidet P-40, 0.1% sodium deoxycholate,

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1% aprotinin and 0.1 mM phenylmethylsulphonyl fluoride. Nuclei were pelleted by centrifugation at $1,000 \times g$, fractionated on a 10% SDS polyacrylamide gels and subsequently analysed by a Western blotting method according to a protocol by Cambridge Research Laboratories. The analysis of tyrosine hydroxylase expression was performed as described in [13] by homogenizing the cells in 300 μ l of 50 mM potassium phosphate buffer, pH 7.4, containing 320 mM sucrose, 1 mM EDTA, 1 mM DTT, leupeptin 100 μ g/ml and 0.5 mM phenylmethylsulphonyl fluoride. The homogenate was centrifuged at $10,000 \times g$ for 10 min and samples from the supernatant were analysed by Western blotting as described above using anti-rabbit TH antibody (Boehringer-Mannheim).

2.6. Isolation and analysis of PKC

PKC was isolated as previously described [4] and analysed by Western blotting method essentially as described above and detected with a monoclonal antibody which recognizes the α and β subtypes of PKC.

2.7. Phosphorylation of the endogenous PKC substrate p80

Cells were grown in 10 cm diameter petri dishes. Carrier free $^{32}\text{PO}_4$ was added at 100 $\mu\text{Ci}/\text{ml}$ 4 h prior to harvest. TPA (100 nM) and/or staurosporine (100 nM) were present during the final 60 min of incubation. The cells were washed rapidly with ice-cold phosphate buffered saline and lysed by addition of 150 μ l buffer, which contained 20 mM

Tris-HCl, pH 7.5, 2 mM EDTA, 2 mM EGTA, 250 mM sucrose, 5 mM dithiothreitol, 0.1 mM phenylmethylsulphonyl fluoride, leupeptin 50 $\mu\text{g}/\text{ml}$ and 0.5% Triton X-100. The samples were boiled and centrifuged at $13,000 \times g$ for 5 min. After addition of Laemmli sample buffer [14] the samples were resolved on a 7.5% SDS-PAGE and p80 phosphorylation was revealed by exposing the dried gel to an X-ray film (Amersham).

3. RESULTS

SH-SY5Y cells treated with staurosporine (25 nM) for 72 h exhibited a differentiated neuronal morphology with long, neurite-like processes (Fig. 1B), which were not detected in untreated cells (Fig. 1A).

Staurosporine at 10 and 25 nM caused a total inhibition of DNA synthesis in SH-SY5Y cells (Fig. 1C). At both concentrations staurosporine was more effective than 10 nM TPA (Fig. 1C), which concentration has previously been shown to be within the optimal TPA concentration range for inhibition of cell growth in these cells [15].

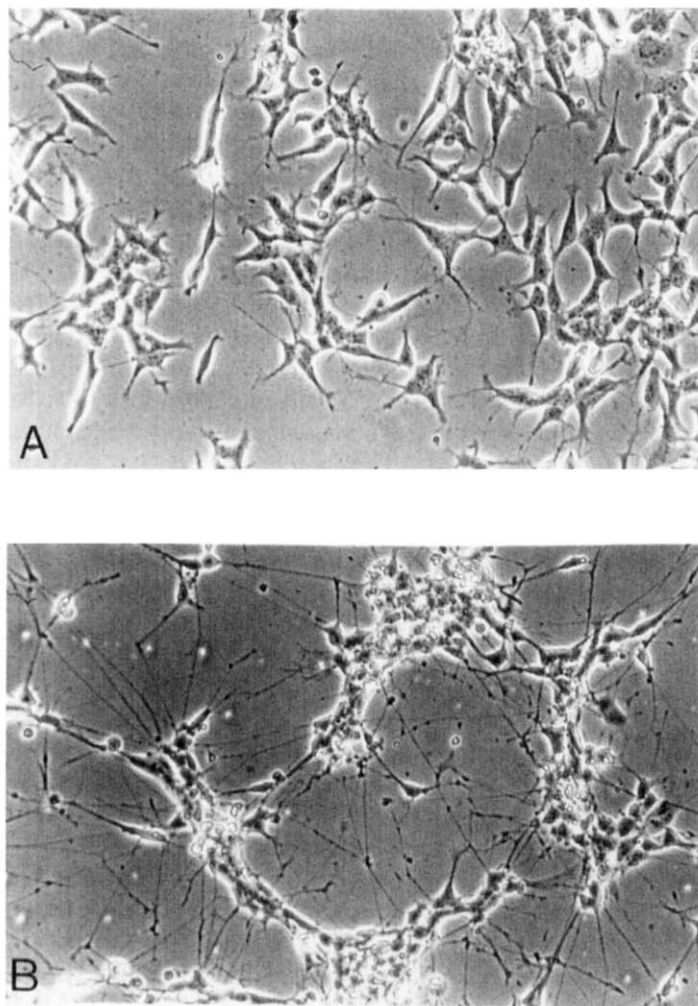
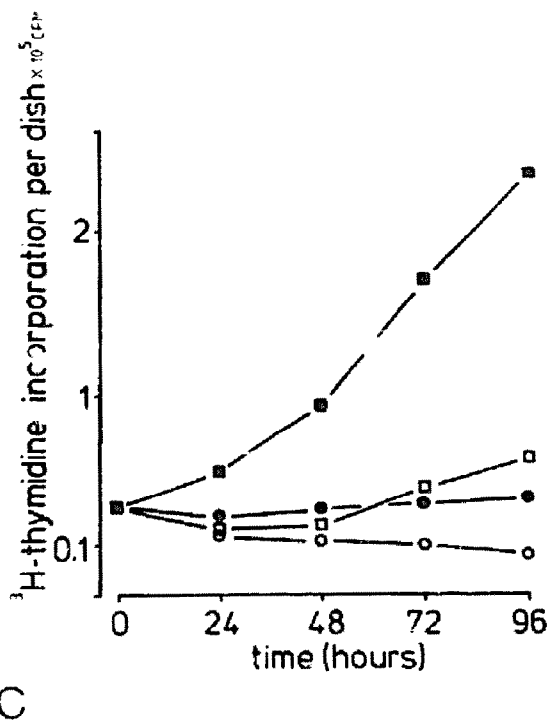


Fig. 1. The effect of staurosporine on morphology and DNA synthesis in SH-SY5Y cells. SH-SY5Y cells were seeded on 5 cm diameter petri dishes. Staurosporine or TPA was added 24 h after plating. (A) control and (B) staurosporine-treated (25 nM) cells were photographed after 72 h of incubation. Phase contrast, $\times 165$. (C) Cells were treated with staurosporine or TPA for the times indicated in the figure and [^3H]thymidine incorporation was measured as described in Materials and Methods. (■) control, (●) 10 nM staurosporine, (□) 25 nM staurosporine, (○) 10 nM TPA.



In untreated SH-SY5Y cells expression of NPY mRNA was observed (Fig. 2A). As shown previously [16] TPA induced an increase in the expression of NPY mRNA (Fig. 2A). Also staurosporine caused an increased expression of NPY mRNA (Fig. 2A). The increase was similar in magnitude with both agents.

Previous results have shown that the TPA-induced neurite outgrowth in SH-SY5Y cells is accompanied by an increased expression of GAP-43 mRNA [17]. Similar results were also obtained in this study where both TPA and staurosporine caused a significant increase in GAP-43 mRNA levels. The stimulation, though, was higher in TPA-treated cells compared to cells which received staurosporine (Fig. 2B).

Expression of tyrosine hydroxylase protein (~60 kDa) in untreated SH-SY5Y cells was below the detection limit of our method (Fig. 2D). A dramatic increase in tyrosine hydroxylase expression was seen in cells treated with 25 nM staurosporine for 72 h (Fig. 2D) while only

a marginal stimulation in the level this enzyme occurred in cells which received TPA.

In SH-SY5Y cells treated with either TPA (10 nM) or staurosporine (25 nM) both induction and down-regulation of c-myc protein expression was observed as analysed by Western blotting (Fig. 3). The c-myc monoclonal antibody 9E10 detected almost equal levels of c-myc protein in SH-SY5Y cells compared to COLO 320 cells, which contain an amplified c-myc gene [18] (Fig. 3). The 9E10 antibody reacted only faintly with proteins isolated from U-1690 cells containing an amplified L-myc gene [19] (Fig. 3). The myc proteins expressed in SH-SY5Y cells were additionally examined with a polyclonal panmyc antibody directed against the highly conserved myc-box portion of the myc family of proteins [20]. An induction of myc proteins by 30 min and a down-regulation by 24 h in the presence of TPA or staurosporine was seen with the panmyc antibody (Fig. 3). The myc protein detected by the panmyc antibody was of the same size as the c-myc protein (62–64 kDa) detected by the 9E10 antibody. The expression of this myc protein also correlated with the respective mRNA levels previously described for c-myc in TPA-treated SH-SY5Y cells [21]. It is thus concluded that the panmyc antibody reacted mainly with the c-myc protein in SH-SY5Y cells, although these cells do express in addition to c-myc at least N-myc [21] and L-myc mRNA (A. Jalava, unpublished observation).

In untreated SH-SY5Y cells almost all immunoreactive PKC protein was in the cytosolic fraction (Fig. 4A). Incubation of the cells in the presence of 25 nM staurosporine for up to 24 h had no effect on the intracellular distribution of PKC (Fig. 4A).

Treatment of SH-SY5Y cells with 100 nM TPA for 60 min resulted in an increased phosphorylation of the p80 substrate (Fig. 4B). When both TPA (100 nM) and staurosporine (100 nM) were added simultaneously a significant reduction in the p80 phosphorylation was observed (Fig. 4B). Staurosporine (100 nM) alone caused a slight decrease in the p80 phosphorylation as compared to control (Fig. 4B).

4. DISCUSSION

The results from the present study show that staurosporine induces a mature neuronal phenotype in SH-SY5Y neuroblastoma cells. This is based on several findings. Staurosporine effectively inhibited DNA synthesis and induced outgrowth of long, neurite-like processes. This was accompanied by an increased mRNA level of GAP-43, a growth-associated protein specific for axonal processes [22]. Although staurosporine was a somewhat weaker inducer of GAP-43 mRNA compared to TPA, this result would indicate that at least some of the processes in staurosporine-treated SH-SY5Y cells are axonal.

Increased tyrosine hydroxylase activity is a well docu-

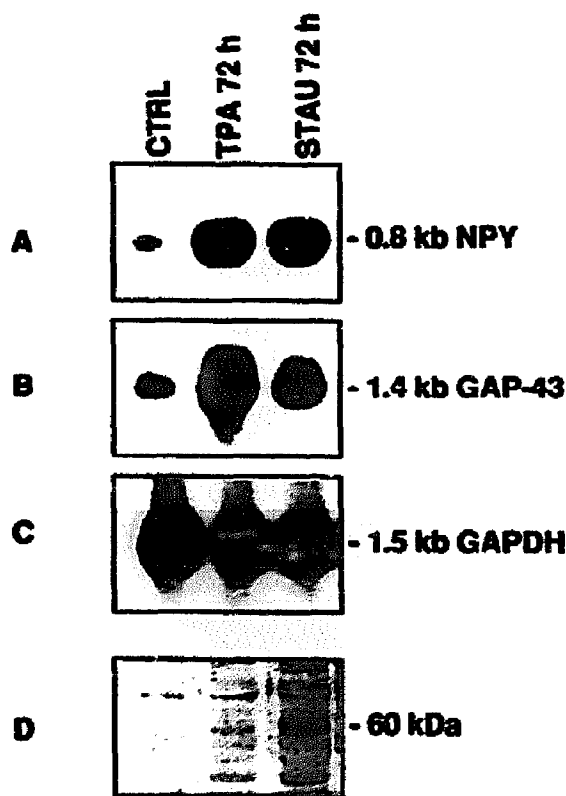


Fig. 2. Induction of markers for neuronal differentiation in SH-SY5Y cells by staurosporine and TPA. SH-SY5Y cells were incubated with 10 nM TPA or 25 nM staurosporine (STAU) as indicated in the figure. In (A–C) poly(A⁺) RNA was isolated and analysed by Northern blotting as described in Materials and Methods. Equal amounts of NPY (A) and GAP-43 (B) mRNA (4 µg), as measured by UV-absorption at 260 nm, were loaded on formaldehyde agarose gel and the actual amounts transferred on the nylon filter were subsequently controlled by probing the filter with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA (C). In (D) proteins from TPA- and staurosporine-treated cells were isolated and quantitated by Western blotting using a polyclonal antibody against tyrosine hydroxylase.

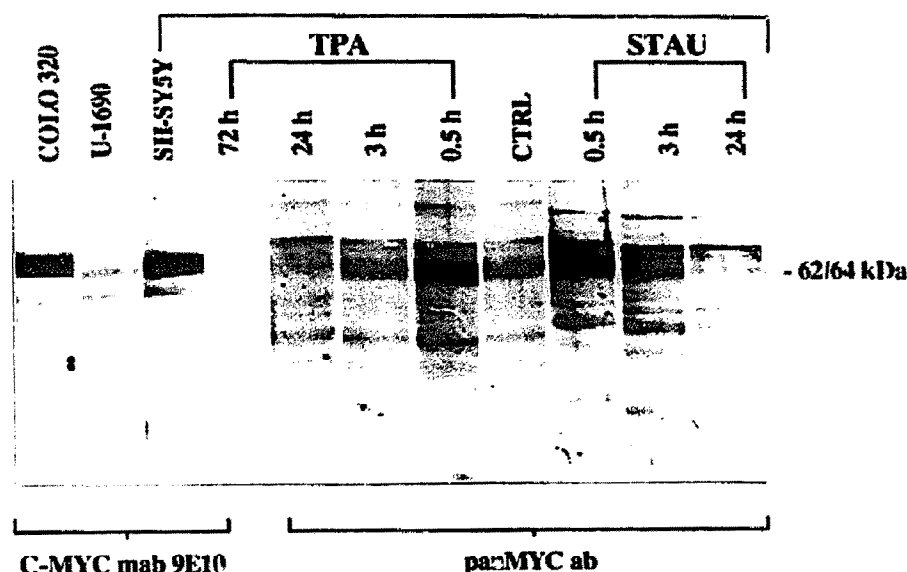


Fig. 3. Expression of myc proteins in TPA- and staurosporine-treated SH-SY5Y cells. Nuclei samples from SH-SY5Y, COLO 320 and U-1690 cells were analysed by Western blotting as described in Materials and Methods. Expression of myc proteins in SH-SY5Y cells treated with TPA or staurosporine (STAU) were analysed by a polyclonal panmyc antibody as indicated in the figure. In comparison, parallel samples from COLO 320, U-1690 and TPA-treated SH-SY5Y cells analysed by a monoclonal antibody to c-myc (9E10) were also included as indicated in the figure.

mented finding in differentiated neuroblastoma cells [23–25]. In TPA-treated SH-SY5Y cells a dramatic increase in noradrenaline content has been reported [1,4]. In this study both TPA and staurosporine stimulated tyrosine hydroxylase protein expression. The increase in staurosporine-treated cells was, however, significantly

higher compared to the levels of tyrosine hydroxylase expressed in TPA-treated cells. Similar increases were observed in noradrenaline content in cells treated with either TPA or staurosporine, the concentration of noradrenaline being significantly higher in staurosporine-treated cells (Jalava and Heikkilä, to be published).

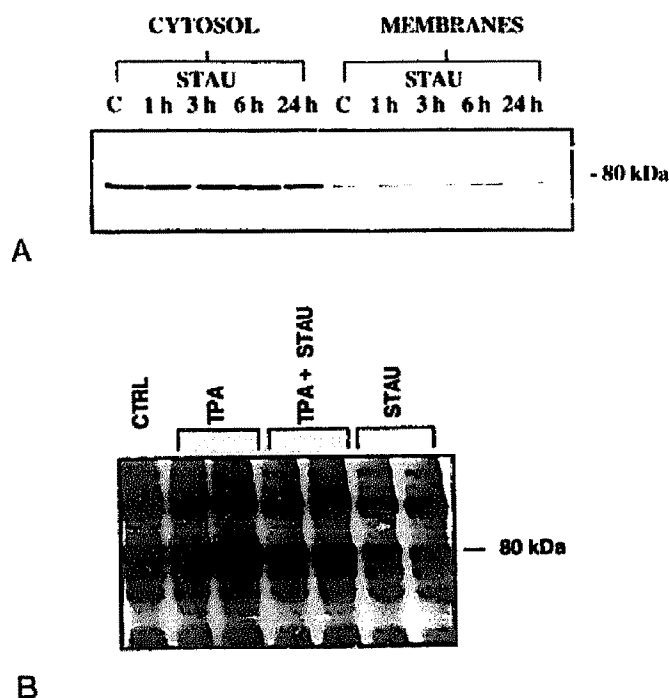


Fig. 4. The effect of staurosporine on intracellular distribution of PKC and p80 phosphorylation in SH-SY5Y cells. (A) The cells were treated with 25 nM staurosporine (STAU) for the times indicated in the figure. Cytosolic and membrane-bound PKC was isolated and analysed by Western blotting as described in Materials and Methods. (B) The cells were labelled with ^{32}P , and treated with 100 nM staurosporine and/or 100 nM TPA for 60 min as described in Materials and Methods. Equal amounts of proteins, as determined by the method of Bradford [33], were separated on a 7.5% SDS-PAGE and the dried gel was subsequently exposed to an X-ray film in order to detect p80 phosphorylation.

NPY is widely expressed in central and peripheral neurones [26,27] as well as in many neuroblastoma cell lines [28]. An induction of NPY mRNA expression was observed both in TPA- and staurosporine-treated SH-SY5Y cells. Since NPY has been shown to co-localize with noradrenaline in sympathetic neurons [29] our results would argue for a noradrenergic phenotype both in staurosporine- and TPA-treated SH-SY5Y cells.

Down-regulation of c-myc proto-oncogene expression has been implicated in in vitro differentiation of several cell types [30–32]. In the present study staurosporine and TPA modulated c-myc protein in a similar fashion as previously reported for c-myc mRNA levels in TPA-treated SH-SY5Y cells [21].

It is obvious from the results of this study that if any, staurosporine only has inhibitory effects on PKC since it failed to stimulate p80 phosphorylation or to translocate PKC to the membrane fraction. Instead, staurosporine inhibited both the TPA-stimulated and basal p80 phosphorylation. It is therefore unlikely that the actions of staurosporine in SH-SY5Y cells are mediated through activation of PKC.

It is also unlikely that the effects of staurosporine are due to inhibition of PKC. The enzyme has been shown to be active in TPA-treated SH-SY5Y cells [17], and down-regulation of PKC by bryostatin 1 blocks the TPA-induced differentiation in these cells [5]. In addition, induction of neurite outgrowth and tyrosine hydroxylase expression, and inhibition of DNA synthesis by staurosporine are not affected by a preceding down-regulation of PKC by bryostatin 1 (Jalava and Heikkilä, to be published).

In conclusion the results of this study show that differentiation of SH-SY5Y human neuroblastoma cells to mature noradrenergic phenotype can be triggered through both PKC-dependent and -independent pathways.

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